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TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY 'S DOCKET NUMBER	
CCP-100	
U S APPLICATION NO (If known, see 37 CFR 1 5	
09/807783	

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/CN99/00157	October 10, 1999	October 19, 1998

TITLE OF INVENTION: LYOPHILIZED HEPATITIS AN ATTENUATED LIVE VACCINE AND STABILIZER THEREOF

APPLICANT(S) FOR DO/EO/US: LIU, Jingye et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. [X] This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. [ ] The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [ ] is attached hereto (required only if not communicated by the International Bureau).
   b. [X] has been communicated by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. [X] An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. [X] is attached hereto.
   b. [ ] has been previously submitted under 35 U.S.C. 154(d)(4).
7. [ ] Amendments to the claims of the International Aplication under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are attached hereto (required only if not communicated by the International Bureau).
   b. [ ] have been communicated by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [ ] have not been made and will not be made.
8. [ ] An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9. [ ] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. [ ] An English lanugage translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. [X] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.AND Attached Form 1449 (1 sheet)
12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [ ] A FIRST preliminary amendment.
14. [ ] A SECOND or SUBSEQUENT preliminary amendment.
15. [ ] A substitute specification.
16. [ ] A change of power of attorney and/or address letter.
17. [ ] A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
18. [ ] A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. [ ] A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. [X] Other items or information: Claims Amended in Accordance with Article 41 of PCT (in English)

U.S. APPLICATION NO (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
09/807783	PCT/CN99/00157	CCP-100

21. [X] The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):	CALCULATIONS PTO USE ONLY
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO $1000.00	$1000.00
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO $860.00	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO $710.00	
International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) $690.00	
International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) $100.00	
ENTER APPROPRIATE BASIC FEE AMOUNT =	$1000.00
Surcharge of **$130.00** for furnishing the oath or declaration later than [ ] 20 [X] 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$ 130.00

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	14 - 20 =	0	x $18.00	$ 0.00
Independent claims	2 - 3 =	0	x $80.00	$ 0.00
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $270.00	$ 270.00
TOTAL OF ABOVE CALCULATIONS =				$1400.00
[ ] Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.			+	$
SUBTOTAL =				$1400.00
Processing fee of **$130.00** for furnishing the English translation later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				$
TOTAL NATIONAL FEE =				$1400.00
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$
TOTAL FEES ENCLOSED =				$1400.00
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a. [ ] A check in the amount of $ _____ to cover the above fees is enclosed.

b. [XX] Please charge my Deposit Account No. 19-0065 in the amount of $ 1400.00 to cover the above fees. A duplicate copy of this sheet is enclosed.

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NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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LYOPHILIZED HEPATITIS AN ATTENUATED LIVE VACCINE AND STABLIZER THEREOF

FIELD OF THE INVENTION

The present invention generally relates to attenuated hepatitis A vaccine, and more particularly to a stabilized lyophilized live hepatitis A vaccine formulation which can be preserved at ambient temperature for a long time, so that eliminated the pressures from transportation, storage and usage of the vaccine without loss of infectivity titers of the vaccine. The present invention further relates to a stabilizer for live lyophilized virus vaccine and its use in producing stabilized lyophilized live vaccine formulations.

BACKGROUND OF THE INVENTION

Hepatitis A is a worldwide distributive acute disease caused by infection with hepatitis A virus (HAV) which is a picornavirus closely related to the poliovirus. Infection is spread by the fecal/oral route and consequently the disease in endemic in areas where hygiene and sanitation standards are lower. Recent reports on epidemical survey show that in developing countries including China, there are as many as 4 millions hepatitis A cases per year. There is frequently large-scale outbreak and rapidly spread in certain regions with a poor social and economic status, especially after various disasters. In these countries or regions, as the high incidence of hepatitis A, some of serious public health and social problems have been encountered. In other hand, in the United States and other developed countries, hepatitis A accounting for approximately 150,000 cases, that is approximately 25% of all clinical hepatitis cases.

Therefore, to successfully immunize against hepatitis A in developing countries as well as in developed countries, it is necessary to vaccinate the entire people, especially entire pediatric populations. So there will be an increasing need for hepatitis A vaccine.

An effective vaccine would be useful for active immunization of populations at high risk. Generally, there are four type of vaccine used for induce a specific neutralizing antibody against challenge with virus or becteria: live vaccine, inactivated vaccine, subunit vaccine (component

vaccine), and recombinant vaccine. In these vaccines, the live attenuated vaccine could elicit a stronger protective response than others, and could have a significant impact on the eradication of the diseases.

U.S Patent Nos.4, 532,215 and 4, 636,469 described, respectively, a strain of wild-type HAV, designated MM-175, initially isolated from feces of a patient, and adapted to passage in vitro in African green monkey kidney culture cell and methods for obtaining a vaccine by serial passaging. Also, CN Patent Nos.85107525 and 92114998 disclosed the preparations of attenuated HAV designated H_2 and L-A-1, respectively.

With regard to live attenuated hepatitis A vaccine, it worthily mentioned the live HAV vaccine based on strain CR-326F (Merck & Co. Inc.) which is under preclinical trials, and the vaccines based on strain H_2 and L-A-1, respectively, which have been licensed for practical use and industrial-scale production in China. Clinical serological studies demonstrated that these live attenuated Hepatitis A vaccines, especially the vaccine prepared from L-A-1 strain of HAV (produced by Changchun Institute of Biological Products, Ministry of Public Health, Changchun, China) evoked high titers of antibody response in most of volunteers received the vaccine after only one dose and no systemic complains were present immediately after vaccination or during long –term follow-up (see CN Patent No.92114988).

However, all of these live hepatitis A vaccines used so far are in form of aqueous suspensions. One of the main disadvantage of live attenuated vaccine is not having satisfactory theremo-stability, even though in the situation of lyophilization at ambient temperature, hence it must be stored and transported under frozen state and used soon after thawing to insure effective vaccination. Hepatitis A virus as well as measles virus are dissatisfactory in both storage stability and heat resistance. For example, live attenuated hepatitis A virus survives only for about 7 days at a temperature of 2-8℃, and the valid storage-term is only about 3-6 months. Therefore, transportation and storage of these vaccine preparations must be finished at a reduced temperature (at a temperature of –20℃ or lower, for example), that is so called "cold chain". As a direct result, the increases of production and transportation cost and user's expense are irrevocable, especially in developing countries and tropical and semitropical areas. So it would be an obstacle to popularization of the worldwide Expanded Program on Immunization (EPI)

founded by World Health Organization (WHO).

For the reasons as described above , eradication of hepatitis A will depend on the ability to provide hepatitis A vaccine formulations having improved theremo-stability. So, there remains a distinct need in the art for live hepatitis vaccine formulations with enhanced storage stability and heat resistance during and after lyophilization.

SUMMARY OF THE INVENTION

In view of these situations as mentioned above, the present inventors have made intensive studies during their production practices to overcome the problems mentioned above and to provide a lyophilized live hepatitis A vaccine with increased thermo-resistance and storage stability. The present inventors have surprisingly found that when added a stabilizer solution to the vaccine stock suspension prepared by a disclosed method (a method described in CN Patent No 92114998, for example), and lyophilized the hepatitis A vaccine formulation comprising, as a virus component, an attenuated live hepatitis A virus and a stabilizer, the storage-term of the hepatitis A virus -containing lyophilized vaccine is extended 3 times longer than non-treated stock viral suspension, so that the "cold chain" pressure and user's expense is decreased greatly to thereby increase the public acceptance of multitude of the vaccine imposed. which is the key to widely applying the vaccine.

It is one object of the present invention to provide a stabilized lyophilized hepatitis A live vaccine formulation comprising a prophylactically effective viral titers of live attenuated hepatitis A virus and stabilizer which can be preserved at ambient temperate for a long time, so that eliminated the "cold chain" pressures from transportation, storage and usage of the vaccine without loss of infective titers of the vaccine, to thereby greatly decrease the expense and relevant cost to ensure effective vaccination against hepatitis A.

In a preferred embodiment of this object of the present invention, wherein said stock suspension of live attenuated hepatitis A virus is prepared by disclosed method based on the wide-type HAV, stain L-A-I.

In another preferred embodiment of this object of the present invention, wherein said stabilizer for lyophilized live hepatitis A virus composed of gelatin, trehalose, one or two amino

acid selected from the group consisting of glutamic acid, aspartic acid, arginine, lysine or alkali metal salts thereof, ascorbic acid, urea, mannitol or sorbitol or both of them, and inositol.

According to a further preferred embodiment of this object of the present invention, the stabilizer for lyophilized live virus vaccine may optionally contains human serum albumin.

In a further preferred embodiment of the stabilizer according to the present invention, the stabilizer for the lyophilized live virus essentially composed of from 0 to 20 grams per liter of human serum albumin, from 5 to 10 grams per liter of gelatin, from 50 to 100 grams per liter of trehalose, from 7.5 to 15 grams per liter of sodium glutamate, from 0.5 to 5.5 grams per liter of ascorbic acid, from 5 to 28 grams per liter of urea, from 2 to 10 grams per liter of mannitol or sorbitol or a mixture of them, and from 4 to 10 grams per liter of inositol .

It is another object of the present invention to provide a method for preparing stabilized lyophilized live hepatitis A vaccine formulation mentioned as above , comprising:

(a) providing a stock suspension of attenuated live Hepatitis A virus;

(b) adding a stabilizer solution to a stock suspension of step (a) at the ratio 1:1(v/v) to obtain a live vaccine formulation comprising prophylactically effective viral titers of live attenuated hepatitis A virus and a stabilizer for attenuated live virus, therein said stabilizer comprises gelatin, thehalose, one or two amino acid selected from the group consisting of glutamic acid, asparitc acid, arginine, lysine or alkali metal salts thereof, ascorhic acid, urea, mannitol or sorbitol or both of them, and inositol;

(c) lyophilizing said vaccine formulation obtained from the step (b).

According to a preferred embodiment of this object of the invention, wherein the lyophilization steps comprises precooling the vaccine formulation to about –20 to –50℃ for about 3 to 6 hours, and then drying the live vaccine formulation by gradually increasing the temperature from –38 to 35℃ in a lyophilizer.

It is a further object of the present invention to provide a stabilizer for lyophilized live virus, said stabilizer essentially composed of gelatin, trehalose, one or two amino acid selected from the group consisting of glutamic acid, aspartic acid, arginine, lysine or alkali metal salts thereof, ascorbic acid, urea, mannitol or sorbitol or both of them, and inositol.

According to a preferred embodiment of this object of the present invention, wherein said

stabilizer essentially composed of from 0 to 20 grams per titer of human serum albumin, from 5 to 10 grams per liter of gelatin, from 50 to 100 grams per liter of trehalose, from 7.5 to 15 grams per liter of sodium glutamate, from 0.5 to 5.5 grams per liter of ascorbic acid, from 5 to 28 grams per liter of urea, from 2 to 10 grams per liter of mannitol or sorbital or a mixture of them, and from 4 to 10 gram per liter of inositol.

According to a further preferred embodiment of this object of the present invention, the stabilizer for lyophilized live virus vaccine may optionally contains human serum albumin.

According to a preferred embodiment of this object of the present invention, therein said stabilizer not only suitable for stabilizing lyophilized hepatitis A live virus, but also used for stabilizing viruses selected from the group consisting of the genus Enterovirus, the genres Papamyovirus, the genus Arbovirus, and the genus Herpevirus against heat inactivation during the period of lyophilization and the period of storage and transportation post-lyophilization to ensure thermo-stability of the lyophilized live vaccine thereby to improve vaculation effectiveness for susceptible population.

DETAIL DESCRIPTION OF THE INVENTION

The present invention is related to lyophilized live vaccine formulations having a increased thermo-stability. Essentially, the vaccine formulations of the present invention are mixture of virus component and stabilizer components, therein the virus component comprises hepatitis A virus or at least one member selected from the genuses Enterovirus, Papamyovirus, Arbovirus and Herpevirus, and the stabilizer components principally comprises gelatin, trehalose ,one or two amino acids selected from the group consisting of glutamic acid ,aspartic acid, arginine , lysine or alkali metal salts thereof, ascorbic acid ,urea, mannitol and/or sorbitol, and inositol. The vaccine stabilizer according to the present invention may optionally contains human serum albumin (HSA) in order to prevent any undesirable enzymolysis of the virus. Upon mixing the two components in a suitable ratio, result in a virus formulation which contains on a gram per liter of final vaccine formulations after lyophilization, from about 0 to 20 grams per liter of HSA, from 5 to 10 grams per liter of gelatin, from 50 to 100 grams per liter of trehalose, from 7.5 to 15 grams per liter of amino acid or alkali metal salts thereof, from 0.5 to 5.5 grams per liter of

ascorbic acid, from 5 to 28 grams per liter of urea, from 2 to 10 grams per liter of mannitol or sorbitol or a mixture of them, and from 4 to 10 grams per liter of inositol.

With respect to a attanuated hepatitis A virus, for example, the HAV stock suspension used for the purpose of the present invention could be prepared from the wide-type strain L-A-1 by the HAV cell-culture adaptation and attenuation method described in detail in CN Patent No 92114998. Briefly, the method composes, cultivating human diploid fibroblast cells in a suitable nutrient medium, for example Eagles's minimal essential medium(MEM) containing 10-15% fetal calf serum (FCS) in roller bottle at 37^0C for 5-8 days. When confluent cell monolayers are formed, the cultured medium is discarded from a culturing vessel and the cells are washed with the same medium or PBS for 3 to 5 times. Then, these cells are inoculated with a seed virus of hepatitis A virus L-A-1 derived from human feces and purified by the method described in Example 1 of CN Patent No.92114998, and then the cells are cultivated in nutrient medium as above at 34-36℃ for 3 to 4 weeks. After the completion of the cultivation, changing the nutrient medium to medium 199 with or without phenol red, and cultivating cells at 34 to 36 ℃ for a additional 4 to 6 days in a cell roller. After harvesting, the cells are sonificated for 3 times, then the cell debris is removed by centrifugation and collecting the resultant supernatant to obtain the desired stock suspension of virus.

The present invention further provides a stabilizer advantigeously used to stabilize a live vaccine , and to protect attenuated live virus against heat-inactivation at ambient temperature for a long period, which is essentially composed of human serum albumin and/or gelatin, trehalose, one or two amino acid selected from the group consisting of glutamic acid, aspartic acid, arginine, lysine or alkali metal salts thereof, ascorbic acid, urea, mannitol and/or sorbitol, and inositol. In a particularly preferred embodiment of the present invention, a stabilizer contains about 0-20g/L of HSA, about 5-10g/L of gelatin, about 7.5-15g/L of sodium glutamate, about 0.5-5.5g/L of ascorbic acid, about 5-28g/L of urea, about 2-10g/L of mannitol and /or sorbitol ,and 4-10g/L of insoitol

Hepatitis A virus is a small picornavirus without outer envelope as well as any of other lipid. Like the majority of live enteroviruses, hepatitis A virus presented in form of aqueous suspension will rapidly loss their ability of replication and propagation, and infectivity. In

absence of suitable stabilizer, it can not effectively protect against hepatitis A infection due to heat inactivation of the virus. Thus eradication of hepatitis A and other epidemic caused by infection with virus will depend on the ability to assure cold storage and transportation of virus vaccine. However, this problem has been circumvented by using vaccine formulations with improved stability according to the present invention.

Vaccine stabilizers are well known in the art as chemical compounds added to a vaccine formulation to enhance vaccine stability during period of the low temperature storage or lyophilization processing or storage post-lyophilization. As described above, the stabilizer aqueous solutions used for formulating and stabilizing the live vaccine of the present invention are preferably composed of a high molecular weight structural additive, a disaccharide, a sugar alcohol and water. The aqueous solution also include one or two amino acids and buffering component. The combination of these components act to preserve the survival and activity of the virus upon freezing and lyophilization and a long storage period subsequent to lyophilization.

It is well known that the high molecular weight structure additive aids in preventing viral aggregation during freezing, and provides structural and nutritional support in the lyophilized or dried state. Within the context of the present invention, the preferred high molecular weight structural additives are human serum albumin and/or gelatin. The amino acids and sugar alcohols function to further preserve viral infectivity upon cooling and thawing of the aqueous suspension. In addition, these components function to preserve viral infectivity during sublimation of cooled aqueous virus suspension during lyophilization and in lyophilized state, and to contribute some buffer capacity The preferred amino acids are arginine and glutamate, and the preferred sugar alcohols are mannitol, sorbitol and inositol. The trehalose, as a preferred disaccharide used in the stabilizer aqueous solution, could be immensely beneficial for stabilizing protein structure of virus to increase heat-resistance and for restoring vigorousness of virus after dehydration. Urea and ascorbic acid, except for the components mentioned above, play an important part in stabilizing hydration state or in maintaining osmotic balance during dehydration period. The buffering component acts to buffer the formulation by maintaining a relatively constant pH which preferably at about 7.0. The preferred buffer is balanced salt solution used for dissolving the chmical componds as above.

These components are added in substantially increasing amount to generate a vaccine stabilizer for combination with viral stock suspensions to thereby generate vaccine formulation for lyophilization which result in a increase in thermo-stability. The preffered component ranges disclosed in this specification allow for generation of vaccine formulations which, among other characteristics, exhibit improved thermo-stability over vaccine formulations known in the art.

The stabilizer of live attenuated vaccine can be formulated by a conventional method, for example by mixing each of components in a suitable vessel, except for preheating the mixture solution of trehalose, gelatin, mannitol and /or sorbitol at about 37℃ for 24 to 48 hours before adding HSA thereto. After 0.5 to 2 hours, mixing the resultant stabilizer with viral stock at about 1.1 (v/v) ratio

It is noteworthy that the ranges of virus stabilizer and final vaccine formulation are presented on a gram per liter basis of the final vaccine formulation. One of ordinary skill in the art will be well aware that changing volume ratio of stabilizer to vaccine may be applied to practice the claimed invention, which in turn will require changes to the concentration of stabilizer components. Therefore, the invention not only limited to the specified 1:1 stabilizer/virus combination to generate the final vaccine formulation for lyophylization.

After the vaccine is formulated with stabilizer and viral stock, the resaltant aqueous suspension should be dried by lyophilization. Briefly, lyophlization involves the steps of precooling the aqueous suspension below the gas transition temperature or below the eutectic point temperature (below –30℃) of the aqueous suspension for about 3 to 6 hours, and then removing water from the cooled suspension by sublimation to form a lyophilized virus. Within one preferred embodiment, aliquots of the formulated attenuated live virus are placed into a refrigerated chamber attached to a freeze dryer. A multistep freeze drying procedure is used to lyophilizing the formulated live vaccine. The temperature is gradually increased from about –38 ℃ to about 35℃ over a period of 10 to 20 hours.

In order to demonstrate improvement in thermal stability and storage stability of live vaccine, the present invention is exemplified by viral potencies, for example by determinating viral titers pre- and post-lyophilization of hepatitis A vaccine, to observe the effectiveness of a stabilizer on the storage stability of live vaccine. The results show that the stabilizer included in

the vaccine formulations of the present invention at a concentration sufficient to stabilize the live virus vaccine against heat inactivation remarkably improved thermal stability of the virus which have been lyophilized and incubated at 37℃ for one week as measured by the log $CC1D_{50}$, as compared with lyophilized control vaccine formulation which is absence of stabilizer.

Further, the present inventors in a comparison experiment found that a similar result can be observed when a stabilizer solution which minus human serum albumin (HSA) component is used for these live virus vaccine mentioned as above if the lyophilization cycle parameters could be suitable adjusted.

The following examples are provided to further illustrate the present invention. It is to be understood, however, that the examples are not to limit scope of the present invention.

EXAMPLE 1

Preparation of stabilizer (I) in accordance with the present invention for lyophilized live virus

Following components are utilized for formulating the stabilizer solution (I):

Component	Amount (g/L)
Human Serum Albumin	10.0
Gelatin	5.5
Trehalose	65.0
Sodium Glutamate	10.0
Urea	20.0
Ascorbic Acid	5.5
Sorbitol	6.6
Inositol	7.5

300 ml of distilled water is added to 5.5 g of purified gelatin, and the resultant mixture is heated for 40 minutes in an autoclave to thereby obtain a solution. Cooling the solution to a temperature of about 30–35℃, and 10.0 g of HSA which has been sterilized by a serial of ultrafiltration is added thereto. In accordance with the formulation as above, corresponding amount of trehalose, sodium glutamate, urea, ascorbic acid, sorbitol and inositol are added in this order, to 500ml of distilled water and thoroughly mixed. Then, the resultant solution is heated for 24 hours at 37℃ After cooling the solution to ambient temperature (about 22–26℃), put the solution into gelatin—HAS solution as above and mixing them thoroughly. Subsequently, distilled water is added thereto to make the total volume to 1000 ml. The pH of the resultant mixture solution is adjusted to about 7.0 by 0.1 N HCL, and is subjected to filtration sterilization once again, to obtain a stabilizer solution(I) which could be used for stabilizing live virus, in accordance with the present invention, to be lyophilization and long-term storage.

EXAMPLE 2

Preparation of stabilizer(II) for lyophilized live vaccine

Following components are utilited for formulating the stabilizer solution(II) in accordance with the present invention, by a similar procedure as described in example 1.

Component	Amount(g/L)
Gelatin	8.5
Trehalose	75.0
Urea	15.5
L-arginine	10.1
Ascorbic acid	3.0
Sorbitol	5.0
Mannitol	5.0
Inositol	4.0

Except that HSA is eliminated from the components because it is very expensive and may cause virus contamination which derived from collected blood sources, and the sodium glutamate is replaced by arginine or alkali metal salt thereof, and added a small amount of inositol thereto.

EXAMPLE 3

Preparation of stabilized lyophilized hepatitis A live vaccine

Essentially, the stock suspension of hepatitis A live vaccine can be prepared by the method described in detail in CN Patent No. 92114998. Briefly, propagating human fetal lung diploid fibroblast cell infected with HAV strain L-A-1 derived from human feces, which strain had been established by Dr. Wang Penfu and his colleague in Changchun Institute of Biological Products, Ministry of Public Health, Changchun, China, in appropriate virus infectious dose in minimum essential medium (MEM) containing 10 % fetal bovine serum (FBS) at 37℃ for 3 to 4 weeks by serial passaging. When the positive infected cells are reached more than 90 % as detected by indirect immunofluorescence technique, the nutrient medium is discarded from culturing vessel, and the residual HSA is washed away by phosphate-buffered saline (PBS). Then, the cultured

medium replaced by medium 199 without phenol red therein, and the infected cells are cultivated for a additional 4 to 6 days. After completion of the culturing and collecting the infected cells by low-speed centrifugation, the infected cells are disrupted by means of 3 cycles of freeze-thawing and sonification. Cellular debris are removed by centrifugation to collect a supernatant as a stock of the vaccine. The stock materiel (suspernatant product) gave a positive result for as antigen by indirect immunofluorescence assay.

After the hepatitis A vaccine is formulated by mixing the stabilizer solution(Ⅰ) prepared in Example 1 and viral stock suspension as above at about 1:1 (v/v) ratio, the resultant vaccine formulation is divided in a small volume (0.5 ml) into 3 ml glass vial. And then the aliquots of the formulated viral vaccine are placed into a freeze dryer (model FS150-SS20C, Hull Co, USA) for multistep lyophilization cycle at –40℃ for 4 hours at first, then the shelf temperature is gradually increased to about –30℃ and maintained primary drying. The shelf temperature is then gradually increased to 32℃ and maintained there for 15 hours to thereby obtain desired lyophilized stabilized hepatitis A vaccine formulation with a very low moisture content.

EXAMPLE 4:

Preparation of stabilized lyophilized measles live vaccine

The stock suspension of attenuated live measles vaccine is prepared in accordance with the Requirement for Measles Vaccine, Live in Requirements for Biological Products. The stock material is mixed with the stabilizer solution (Ⅱ) prepared in Example 2 at 1:1 (v/v) ratio to obtain measles live vaccine formulation. The vaccine formulation is precooled at –40℃ for 5 hours, and then the formulation is subjected a drying treatment at about –35℃ to 34 ℃ for 14 hours to result in the lyophilized stabilized measles live vaccine.

EXAMPLE 5

Storage stability testing of lyophilized hepatitis A live vaccine

The samples of lyophilized hepatitis A live vaccine from different lots of viral formulation prepared in Example 3 which are stored at 2--8℃ for 3 to 12 months, are ten-fold serially diluted, then the sample at 10^{-2} to 10^{-7} dilution is used for determinating the viral titers every three months.

The vaccine formulation from the same lot and lyophilized by the same lyophilization cycle parameters but no stabilizer therein is used as a control sample. After adding distilled water for injection to the lyophilized vaccine for reconstitution, the resultant suspention containing the live vaccine and stabilizer is subjected to testing for storage-stability by determinating viral titers ($CCID_{50}$) using conventional enzyme linked immunoadsorbant assay (ELISA) and indirect-immunofluorescence assay (IF). The results of the testing reveal that the 5 lots of samples which are lyophilized in the presence of stabilizer solution exhibited a higher infectious titer in the range from about 6.33 to about 6.50 log $CCID_{50}$, whereas the 5 lots of control samples which are lyophilized in the absence of stabilizer solution exhibited a remarkable decreased infectious titers in the range from about 1.33 to 2.33 log $CCID_{50}$.

In another experiment, the lyophilized virus samples from same lot of vaccine formulations are stored at 2-8℃, 25℃ and 37℃, respectively and each of the samples are sampled everyday and subjected to testing for storage stability in terms of lowest valid storage periods by detecting the log $CCID_{50}$ values. Live vaccine in form of aqueous suspension is compared to lyophilized vaccine formulation.

The results are summarized in Table 1 and Table 2 below, respectively.

Table 1 Storage stability test of lyophilized live hepatitis A vaccine formulation with stabilizer

Lot number of sample	Months of storing at 2 –8℃				
	0	3	6	9	12
1	6.50*	6.67	6.67	6.50	6.50
2	6.67	6.50	6.67	6.50	6.67
3	6.50	6.50	6.33	6.50	6.50
4	6.50	6.67	6.67	6.50	6.33
5	6.33	6.50	6.50	6.50	6.33
control sample	2.33	1.75	1.50	1.50	1.33

*Infective titers of the virus (log $CCID_{50}$/ml)

Table 2 Comparison of stability of hepatitis A live vaccine

Temperature of storage	Lowest valid storage period (days)	
	Aqueous suspension	Lyophilized formulation
2—8℃	180	360
25℃	7	90
37℃	1	7

It can be seen from the results showed in table 1 and table 2 as above, that the stabilizer for lyophilized live vaccine of the present invention greatly increased thermo-stability measured as the log $CCID_{50}$, due to it stabilizes structure of viral protein and nucleic acid, and effectively maintains viral potency of the vaccine under the conditions of enhanced temperature and osmotic strength.

EXAMPLE 6

The Immunogenicity and Safety Testing of the Lyophilized Hepatitis A Live

Vaccine

The lyophilized hepatitis A live vaccine formulation prepared in accordance with the method in Example 1 which has been stored at about 25℃ for 30 days is intravenously inoculated into healthy rhesus monkeys (each group comparing 5 animals). Every two weeks bled for 8 weeks and checked for abnormally elevated serum enzymes (GPT) levels and the titers of anti-HAV antibody. Abnormal elevations of enzymes (more than 25U/ml) would indicate the presence of hepatitis A disease in the animals and the presence of antibody would show protection (Table 3). In this experiment, a fresh vaccine preparation in initial state from the same lot but which is unlyophilized and without stabilizers therein is used as a control sample. All of the animals are received a $10^{6.5}$ $CCID_{50}$ viral infectious dose (1.0 ml of the stock). The results are summarized in Table 3 below.

Table 3 Serum GPT abnormal elevation and antibody response of animals before and after inoculation with the lyophilized hepatitis A vaccine.

Lots number Of vaccine	Abnormal elevation of SGPT*				anti-HAV IgG Ab**				anti-HAV IgM Ab**			
	0	2	4	8(w)	0	2	4	8(w)	0	2	4	8(w)
1	0/5	0/5	0/5	0/5	0	60	100	100	0	60	40	0
2	0/5	0/5	0/5	0/5	0	40	100	100	0	80	40	0
3	0/5	0/5	0/5	0/5	0	60	80	100	0	60	40	20
4	0/5	0/5	0/5	0/5	0	60	60	100	0	80	20	0

* Serum GPT value $\geqslant$ 25 U/ml is considered to be abnormal elevation of the enzyme.

** The data given in the table represent percentage of seroconvertion for 5 animals.

It is can be seen from the results showed in Table 3, that all rhesus monkeys developed anti-HAV protective antibody and more than 80% of seroconverted animals also developed IgM anti-HAV at about two weeks after inoculation. In the other hand, none of the rhesus monkeys have elevation of liver enzymes atributable to the vaccination. All values for these higher primates is within normal limits. It indicates no biochemical evidence of hepatitis. These results

exhibited comparable immunogenicity and safety with control samples, and show that the lyophilized live vaccine formulation of the present invention which has been stored for 30 days at ambient temperature still maintains a similar immunogenicity and safety like in its initial state.

EXAMPLE 7

Storage-stability of lyophilized measles live vaccine

Storage-stability testing of measles live vaccines pro- and post-lyophilization stored at 2–8 ℃ and 37℃, respectively, are performed in substantially the same manner as described in Example 5

The results show that, in the presence of stabilier of the present invention, 5 lots of the measles vaccine exhibited a slightly decreased themostability subsequent to lyophilization, that is less than 0 5 log loss in comparison to the control vaccine in initial state. Further, the lyophilized measles vaccine sample which is stored at 2–8℃ for 15 months and at 37℃ for 4 weeks lossed their $CCID_{50}$ are less than 0.5 and 1.0, respectively.

Amended claims to be filed with the USPTO

CLAIMS

What is claimed is:

1. A stabilized lyophilized hepatitis A live vaccine formulation comprising a prophylactically effective titers of live attenuated hepatitis A virus and a stabilizer, wherein said stabilizer being present in the vaccne formulation at a concentration sufficient to stabilizer the hepatitis A virus against heat inactivation.

2. A stabilized lyophilized hepatitis A live vaccine formulation according to claim 1, wherein said live attenuated hepatitis A virus is prepared by disclosed method based on the wide-type HAV, stain L-A-I.

3. A stabilized lyophilized hepatitis A live vaccine formulation according to claim 1, wherein said stabilizer for lyophilized live hepatitis A virus composed of gelatin, trehalose, one or two animo acid selected from the group consisting of glutamic acid, aspartic acid, arginine, lysine or alkali metal salts thereof, ascorbic acid, urea, mannitol or sorbitol or a mixture of them, and inositol.

4. A stabilized lyophilized hepatitis A live vaccine formulation according to claim 1, wherein said stabilizer for lyophilized live virus vaccine may optionally contains human serum albumin.

5. A stabilized lyophilized hepatitis A live vaccine formulation according to claim 1, wherein said stabilizer for the lyophilized live virus essentially composed of from 0 to 20 grams per liter of human serum albumin, from 5 to 10 grams per liter of gelatin, from 50 to 100 grams per liter of trehalose, from 7.5 to 15 grams per liter of sodium glutamate, from 0.5 to 5.5 grams per liter of ascorbic acid, from 5 to 28 grams per liter of urea, from 2 to 10 grams per liter of mannitol or sorbitol,

and from 4 to 10 grams per liter of inositol .

6. A method of preparing stabilized lyophilized live hepatitis A vaccine formulation according to any one of the claims 1 to 5, comprising:

(a) providing a stock suspension of attenuated live Hepatitis A virus;

(b) adding a stabilizer solution to a stock suspension of attenuated live hepatitis A virus obtained from step (a) at the ratio 1:1(v/v) to obtain a live vaccine formulation comprising prophylactically effective titers of live attenuated hepatitis A virus and a stabilizer for attenuated live virus, wherein said stabilizer comprises gelatin, thehalose, one or two amino acid selected from the group consisting of glutamic acid, asparitc acid, arginine, lysine or alkali metal salts thereof, ascorhic acid, urea, mannitol or sorbitol or a mixture of them, and inositol.

(c) lyophilizing said vaccine formulation obtained from the step (b).

7. A stabilizer for lyophilized live virus, wherein said stabilizer essentially composed of gelatin, trehalose, one or two amino acid selected from the group consisting of glutamic acid, aspartic acid, arginine, lysine or alkali metal salts thereof, ascorbic acid, urea, mannitol or sorbitol or a mixture of them, and inositol.

8. A stabilizer according to claim 7, wherein said stabilizer essentially composed of from 0 to 20 grams per titer of human serum albumin, from 5 to 10 grams per liter of gelatin, from 50 to 100 grams per liter of trehalose, from 7.5 to 15 grams per liter of sodium glutamate, from 0.5 to 5.5 grams per liter of ascorbic acid, from 5 to 28 grams per liter of urea, from 2 to 10 grams per liter of mannitol or sorbital, and from 4 to 10 grams per liter of inositol.

9. A stabilizer according to claim 7, wherein said stabilizer for

lyophilized live virus vaccine may optionally contains human serum albumin.

10. A stabilizer according to claim 7, wherein said stabilizer not only suitable for stabilizing lyophilized hepatitis A live virus, but also used for protecting viruses selected from the group consisting of the genus Enterovirus, the genres Papamyovirus, the genus Arbovirus, and the genus Herpevirus against heat inactivation during the period of lyophilization and the period of storage and transportation post-lyophilization to ensure thermo-stability of the lyophilized live vaccine thereby to improve vaculation efficacy for susceptible population.

ABSTRACT

The present invention relates to hepatitis A vaccine, especially to a lyophilized attenuated hepatitis A vaccine which can be stored at ambient temperature for a long time, and to a method for producing the same. The present invention further relates to a stabilizer for lyophilized live vaccine and its use in improving thermostability of lyophilized live vaccine during lyophilization precessing and storage period after lyophilization.

Docket No. CCP-100 Declaration and Power of Attorney for Patent Application

專利申請聲明及委託書

Chinese Language Declaration

中文聲明

作為下述發明者，我在此宣告：

As a below named inventor, I hereby declare that:

我的住址、郵局地址和國籍均列在我名下，

My residence, post office address and citizenship are as stated next to my name.

我相信我是首創的、第一個和唯一的發明者(如只列出一人姓名)或是首創的、首位共同發明者(如列出數人姓名)，我提出作為專利申請權利要求的題目如下

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

LYOPHILIZED HEPATITIS AN ATTENUATED LIVE VACCINE AND STABILIZER THEREOF

如不在下面小方格中打叉則須將說明書附此：

the specification of which is attached hereto unless the following box is checked:

☐ 以美國申請號碼或PCT國際申請號碼 _____
立案于 _____
修正于(如適用) _____

☒ was filed on April 17, 2001 as United States Application Number or PCT International Application Number 09/807,783 _____ and was amended on _____ (if applicable).

我在此聲明我已閱畢并理解上述說明書的內容，包括上述任何修正案所修正的權利要求。

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

按照聯邦法規第三十七節第一．五六條，我有責任提供支持專利權的實質性資料。

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56.

Docket No. CCP-100

Chinese Language Declaration

我申請享受按照美國法規第三十五節第一百一十九條(a)-(d)項或第365條(b)項列出的以下任何外國專利申請書或發明者證書或第365條(a)項列出任何PCT國際申請指定至少在美國以外的任何一個國家的外國優先權，並確認下列方格內打記號，具有優先權申請前立案日期的、任何外國專利申請書或發明者證書或是PCT國際申請書。

I hereby claim foreign priority under Title 35, United States Code, § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed.

不要求優先權
Priority Not Claimed

國外優先申請書

(號碼) (Number)	(國名) (Country)	(申請日/月/年) (Day/Month/Year Filed)	
PCT/CN99/00157	PCT	Oct. 10, 1999	☐
98120633	China	Oct. 19, 1998	☐
			☐

我申請享受被美國法規第35節119(e)列出的以下任何美國臨時申請書的利益。

I hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) listed below.

(申請順序號碼) (Application No.)	(申請日期) (Filing Date)

我申請享受按照美國法規第三十五節一百二十條或365條(c)項列出任何PCT國際申請所指定的美國列出的以下任何美國申請書的利益，如果此申請書中提出的每項權利要求的題目未按美國法規或是PCT國際申請第三十五節第一百二十條第一段的要求在以前的美國申請書中披露，則我有責任按照聯邦法規第三十七節第一·五六(甲)條提供支持專利權的實質性資料，這一法規條文生效于以前申請的立案日期之後，但在美國或PCT國際申請立案日期之前。

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s), or § 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

(申請順序號碼) (Application No.)	(申請日期) (Filing Date)	(狀況) (已獲專利權、申請中、取消)	(Status) (patented, pending, abandoned)

我在此聲明根據我所知而作的所有聲明都真實無誤，所有有關資料和信息的聲明也真實無誤；我還知道，按照美國法規第十八節第一千零一項，任何蓄意偽造的聲明都將受到罰款或監禁，或同時受到兩種懲罰。這類蓄意偽造的聲明將危及此申請書或任何已頒發專利的效力。

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application of any patent issued thereon.

Docket No. CCP-100

Chinese Language Declaration

委託書：

以列名發明者的身份，我在此指定下列律師和/或代理人執行此申請並從事與專利商標公署有關的所有業務(列出姓名和註冊號碼)：

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